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SMUFD D/A ltr, 18 Feb 1972

TRANSLATION NO. [illegible]

DATE: [illegible]





DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

ANNALES DE L'INSTITUT PASTEUR T-722-4

# THE MECHANISM OF AGGLUTINATION

by Dr. Jules BORDET

(Laboratory work by M. Metchnikoff)

The term "agglutination phenomenon" usually refers to the fact that, under the influence of a specific serum, the microbes in homogenized suspension in a liquid such as bouillón or a 0.7% sodium chloride solution, form flakes which settle to the bottom of the vessel. In 1895 we saw the first example of this phenomenon, which showed that the cholera vibrios, diluted with normal saline solution, become immobilized under the action of a very weak dose of cholera serum, whether cool or preheated to 55° or 60°, and agglomerate rapidly in lumps which float in the liquid.[1]

This fact of agglutination should be considered from various points of view. In the first place, it should be studied in itself, independently of its physiological significance. Seen from that aspect, the study of agglutination touches the domains of both physics and chemistry. It comes within the framework of physiology since it tries to specify the significance of the phenomenon in immunity, knowing that it plays a role in the defense of the organisms, recognizing the cells that are capable of secreting the substances which are poured into the serum and lend their particular properties to it.

The question of the mechanism of agglutination will be the subject of this work, and we will rapidly outline the principal theories which have been proposed to explain the phenomenon. We will immediately make the remark that, to satisfy the mind, all theories of this type should have a sufficiently general scope and not limit us merely to an understanding of the agglutination undergone by microbes. They are far from being the only elements susceptible of agglumerating under the action of a serum. We had thought that it would be appropriate to

1. The leucocytes and the active properties of serum in vaccinated animals. The Annales, 1895, p. 496 and 498.

move from the agglutination of microbes to what happens to red corpuscles under the influence of a serum coming from an animal of a different species. We have shown moreover that one can, by repeated injections of defibrinated blood, obtain a specific serum, endowed, as far as the corpuscles are concerned, with high-energy agglutinating properties.[1] Therefore, to be acceptible, all explanations should be applied as much to the agglutination of corpuscles as to that of microbes and even, as the reader will see farther on, to the agglutination which can be undergone by casein particles in suspension in milk.

That observation made, the following are, in chronological order, the various hypotheses which have been proposed:

1. Gruber's Hypothesis: Mr. Gruber admits that the agglutinin rather deeply alters the substance of the microbe. It has the effect of making the membrane of the microorganism viscous. This special viscous state of the peripheral layer causes the microbes to adhere to each other and explains their joining into cohesive lumps.

This concept makes it easy to understand how the microbes, once joined, continue to stay associated, but it does not indicate at all why they join each other to form lumps. In the explanation of the fact, it places a very great and even quite exclusive importance on the organic structure of elements susceptible to agglutination, without even suggesting that a single part of the phenomenon could be under the control of physical laws. Since it rests entirely on the existence of a change in the membrane, a swelling accompanied by the production of an adhesive material, it does not explain the agglutination of inorganic particles, particles of mineral matter, for example. Thus it rules out any rapprochement between the phenomenon of microbe agglutination and that of the flaking of chemical precipitates produced in a liquid.

2. Bordet's Hypothesis: The idea that we had of the phenomenon in 1896 follows rather a contrary trend. We had the impression, observing agglutination of the cholera vibrio under the influence of an active serum, that it concerned an action in which the microbes only played a passive role, in which their vitality didn't enter into the matter at all. The rapid immobilization they underwent, the existence of agglutination in the red corpuscles, which are inert elements, caused the consideration of mobility to be excluded. The fact of microbic passivity was demonstrated to us when we saw agglutination work on dead microbes. On the other hand, Mr. Gruber's hypothesis

1. On the agglomeration and dissolution of red corpuscles by serum. Annales, 1898, p. 896.

raises certain objections which we will return to further on. It seems to us that the fact of agglutination "falls instead in the area of molecular physics. Slight influences suffice to cause the aggregation of chemical precipitates that were previously spread throughout a liquid. It is probable that the serum, in acting on the microbes, changes the relations of molecular attraction between the microbes and the liquid medium."[1]

It is clear that this interpretation does not allow, any more than Mr. Gruber's does, a really deep explanation of the phenomenon. It merely makes a rapproachement between particles of very different natures, microbes, corpuscles, chemical precipitates--which can be found in suspension in liquids and are susceptible of coming together in clumps under the influence of certain causes. In opposition to the concept of Mr. Gruber, this viewpoint implies the existence of analogies in the process of agglutination, namely, the nature of the elements which undergo it; it admits that the laws of physics play a predominant role in the phenomenon.

Yet it is easy to understand. Does the hypothesis mean that under the influence of the agglutinin, and in all phases of the phenomenon, the microbes act like particles of whatever nature? Evidently not. The agglutinins are specific; there is no longer any doubt that they do not act directly on the microbes; these latter, in effect, just as one has been able to see from the first findings on the phenomenon, immobilize rapidly under their influence. In the first stages of the phenomenon the action of the agglutinin takes into account -- it is scarcely necessary to emphasize -- the biological nature, the special organic constitution of the element which it affects: it takes it so into account that it acts on certain microbes and not on others. But it is enough that the active substance has produced modifications, however slight, restricted to changing the ratio of molecular adhesion between the microbes and the liquid so that the affected microbes later form clumps.

Ever since, after the hypothesis which it affects, the biological nature of the elements never entered the picture; the microbes agglutinated thereafter in accordance with physical laws which are also applicable to certain non-organic particles which agglomerate -- without it being necessary to invoke the pressence of an adhesive material, of sticky and viscous membranes to explain the formation of clumps and the adherence of the microbes. Mr. Gruber's hypothesis excludes the participation of physical laws; the second makes them largely intervene, at least

1. Method of action of preventive sera. The Annales, April 1896. p. 227 footnote.

during that phase of the phenomenon in which the microbes are still scattered, but when they are touched by the agglutinin they come together in the phenomenon of agglutination.

These two hypotheses formulated at the beginning of the studies on agglutination could not then be supported on a solid base. Facts were needed. One important experimental notion was provided by Mr. Kraus.

Mr. Kraus[1] shows that if one mixes a serum from animals vaccinated against the cholera vibrio with a clear and filtered culture of that vibrio, a precipitate is produced in the liquid. This reaction is specific and is not produced if, instead of the cholera serum, any other serum were used. The precipitate formed possesses the property of agglomerating rapidly in flakes which look to be similar to the real flakes of agglutinated microbes. Mr. Kraus showed that the same fact is also verified by other microbes (typhoid fever) and by other sera.

Let us hasten to say that these experiments appear to corroborate a priori the second of the two hypotheses which we have cited. They seem to argue clearly against the hypothesis of Mr. Gruber, which recognizes a structural modification of the organic element as the unique cause of agglutination. They show, in effect, that one can obtain the phenomenon of flaky precipitation very similar to real agglutination by adding to the serum a liquid containing not only organic microbes but simply material which comes from microbic disintegration. The fact observed by Mr. Kraus thus appears to tend to refute the theory formulated by Mr. Gruber.

3. Nicolle's[2] Hypothesis: That, however, has not been the impression of Mr. Nicolle. Mr. Nicolle has confirmed the results obtained by Kraus, and admits that the agglutinin precipitates the agglutinable (or agglutinated) substance of the microbes.[3] It also admits that the agglutinable matter, which, in old cultures, can be diffused in the liquid medium, is found in abundance in the membrane or peripheral layer of the microbes when they are young and in good condition.

1. Kraus, K. K.,Gesellschaft der Aerzte in Wien (Society of Doctors in Vienna), 30 April 1897; and Wiener Klinische Wochenschr. (Viennese Clinical Weekly), 12 August 1897, No. 32.

2. The Annales, March 1898.

3. This is more than a simple translation, in current language, of Mr. Kraus' own experiment; the phrase signifies that he could admit -- a bit prematurely, perhaps -- that the precipitated substance is really that which plays the largest role in microbe agglutination which, in other words, represents the element in the microbe which is sensitive to agglutinin.

This peripheral layer, enclosing the substance which the agglutinin is capable of reaching and precipitating, itself becomes sensitive to the influence of the agglutinin; under the action of this latter, the external layer of the microbe "swells, becomes visible, and is joined to the external layers of neighboring individual microbes. Our opinion about the inner nature of the phenomenon of agglutination thus is quite close to that offered by Mr. Gruber, which only Mr. Roger defended after him. We think that agglutination consists in the coagulation and coalescence of the external layers of microbes which are agglutinable under the influence of an agglutinant serum."[1]

As Mr. Nicolle points out, one can see a connection between the experiment of Mr. Kraus and Gruber's theory. But the connection thus established, dealing with the union thus posed, and on which the entire merit of the idea rests, is precisely the weak point of the reasoning and rather escapes comprehension. Why, in effect, does the precipittation of an agglutinable substance within the external layer of the microbe (a precipitation the existance of which we do not want to deny too hastily) bring about a swelling, a viscosity capable of assuring coalescence or welding of the external layers of the microbes present?

As is known, this interpretation is as unconcerned about bringing in, through the explanation of fact, the notion of physical laws, laws of molecular adhesion as is that of Mr. Gruber, with which it is connected. It does not further foresee the possibility of a rapproachement between the fact of the aggregation of certain chemical precipitates and the fact of microbe agglutination, which rests exclusively, for a complete explanation of the phenomenon, on the existence of a membrane, an external layer or a ciliated skin susceptible of swelling and of welding.

In his articles in the Annales, Mr. Nicolle described a very curious experiment. Mr. Nicolle showed that the precipitate provoked by the active serum in the culture filtrate has the property of drawing into its agglomeration the inert particles which had previously been added to the liquid, talcum powder, for example, which thus form clumps.

The experiment is interesting, but we think nonetheless that one cannot attribute any real importance to it in explaining the phenomenon of agglutination, of which it gives us only an artificial image. These talc particles which cluster are mechanically drawn toward each other, incorporated into and carried along by a precipitate in the process of binding. According importance to this non-specific phenomenon, whose resemblance to true agglutination appears a priori superficial, would

1. Loc. cit, p. 191

necessarily be a mistake -- and then we would find ourselves in the presence of a new interpretation -- to admit that the so-called agglutination is also due to the fact that it forms an extramicrobial precipitate which retracts and agglutinates, enclosing and incorporating the microbes, thus forcing them to come together and finally adhere to one another. In fact, this opinion was held by Mr. Paltauf[1] before the work of Mr. Nicolle. Mr. Dineur discussed it in a recent article[2]; moreover he fought it with the aid of appropriate arguments. We must cite this idea in the list of hypotheses held:

4. Paltauf's Hypothesis -- The agglutination of microbes must be due to the fact that they are mechanically drawn into the mesh of a coagulum born within the liquid medium, outside of the microbes, and resulting from the reaction of agglutinin on the agglutinable microbial matter.

5. Dineur's Hypothesis: -- For Mr. Dineur, agglutination is due to the formation of an adhesive matter which assures the adherence of the microbes to each other. But this adhesive matter is formed specially on the cilia. Mr. Dineur also attributes an altogether essential importance to the presence of cilia in the agglutination, which would be produced by the adhesion and tangling of the cilia.

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We have reviewed, in the foregoing pages, the various interpretations proposed, insisting upon their precise meaning and tendencies. But we have barely scratched the surface of the experimental facts which corroborate them or raise doubts about them. It is the examination of these facts that we are going to take up here. Today they are too numerous to permit a well documented discussion of the various theories.

Among the interpretations held there is one which manifestly does not fit the facts, without which a prolonged examination would be needed. That is Mr. Dineur's idea which attributes primary importance to the existence of cilia. Such an opinion would not be satisfactory, considering that the agglutination of microbes which have no cilia and even that of elements, red corpuscles, casein particles, even less suspect of possessing such appendages, have already been established.

It is true that Mr. Dineur insists equally on the production -- under the influence of agglutinin -- of an adhesive substance which allows the microbes to form into clumps. This concurs with Mr. Gruber's interpretation, which we shall take up later.

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1. PALTAUF, Wiener Klin. Wochenschrift (Vienna Clinical Weekly), 1897.

2. DINEUR, Research on the mechanism of agglutination of the typhus bacillus. Bulletin of the Belgian Academy of Medicine, 1898, p. 652.

Mr. Paltauf's interpretation, which explains the agglutination of microbes by the retraction of a precipitate (Kraus precipitate) born in the liquid and able to draw the microbes into their own cluster, comes up against serious objections. In the first place, this phenomenon of the Kraus precipitate is not absolutely constant; when it exists the precipitate is never very abundant and is produced so slowly that it is difficult to consider it preexistant to the energetic and rapid agglutination of the microbes, as being the determining cause. Further, observers examining the agglutinated microbes have thus far not been able to establish the existence of a coagulum around them. Mr. Dineur has never succeeded, in spite of his repeated efforts: he remarks with reason that if this coagulum existed, if it really incorported the microbes, there would be ample opportunity to reveal it, seeing that it is colored, as Mr. Nicolle has shown, by the basic colors.

Other remarks could also be presented. Rabbits which have received many interperitoneal injections of defibrinated chicken blood, provide a serum which possesses an energetic dissolvant and agglutinant power for chicken red corpuscles. But this active serum possesses yet another property. Added to chicken serum, it produces a preciptiate in this liquid which very slowly increases and agglomerates in flakes. This property possessed by the serum of treated animals, of producing a precipitate in a serum identical to that with which they were injected, has been established, for the first time, by M. Tchistovitch during his recent research at the Pasteur Institute. Mr. Tchistovitch observed that the serum of rabbits injected at repeated intervals with eel serum, clouds the liquid; he found the same to be true when studying the serum of rabbits that had been injected with horse serum: of course, it was this liquid that was clouded. The precipitates obtained by Mr. Tchistovitch were, as has been seen, soluble in small amounts of alkalis (potassium, soda, AZII[3]) /no footnote given in text/, a fact which we have been able to verify ourselves in working on our rabbit serum which is active for chicken blood.

It seems legitimate to compare these phenomena with those which have attracted the attention of Mr. Kraus. The specific serum extracted from animals which have themselves been injected with a new-drawn blood or serum, clouds whichever new serum is identical with that which was used for the innoculations. The serum extracted from animals injected with one culture clouds the liquid of a culture similar to the one that was employed in the vaccination. The precipitate of which we speak is thus to the Kraus precipitate what the agglutination of corpuscles is to microbe agglutination: the comparison is therefore justified. Now, experience has shown that the existence of these precipitates is not indispensable to the production of a strong agglutination, and it is not even correlative. The serum of a rabbit subjected to repeated

injections of chicken blood presents the property of agglutinizing (and dissolving) the corpuscles and precipitating the serum of the latter animal. But it also produces a precipitate in pigeon serum. Thus one would expect, correlatively, that the serum in question would also strongly agglutinate pigeon corpuscles. It only agglutinates them weakly, without showing any more activity in that regard than new-drawn rabbit serum which, when mixed with pigeon serum, remains entirely clear. This agglomeration is clearly inferior in intensity to that undergone, for example, by new-drawn rabbit corpuscles placed in contact with new-drawn chicken serum, which is not accompained by any precipitation. On the other hand, guinea pigs subjected to frequent injections of defibrinated rabbit blood give us a serum which, for rabbit corpuscles, shows a very intense agglomerant power but which does not produce any visible clouding of the rabbit serum. There is thus no obligatory parallel between the appearance of precipitates and the exitance of an intense agglomeration. The opinion which considers the formation of such precipitates as the sine qua non condition of agglutination, does not seem to us to be sustainable.

We come to the hypothesis of Mr. Gruber, which has raised criticism from the time it appeared. It is easy enough to conceive that the glairous substance produced by the microbe casing holds the clustered microbes together, but it is less clearly seen because they come together very rapidly. Neither Mr. Pfeiffer nor we ourselves were able to find any trace, by microscopic examination of live, colored microbes or corpuscles, of the morphological change which that hypothesis implied.

Mr. Trumpp[1], however, thinks he has verified the change in cholera vibrios under the influence of agglutination. But he observed swelling of the microbes in the body fluids which, while they contained agglutinin, also contained alexin (or lysin) the bacteria-and corpuscle-killing material which is eliminated at a temperature of 55°. Alexin is an energetic alterant of the vibrios as well as of the corpuscles; it can dissolve the latter and produce great changes in the microbes, cause them to swell, transform them into granules and even destroy them. It is evident that Mr. Trumpp would have to use liquids which had previously been divested of the alexin they contained in order to appreciate the influence of the agglutinin itself.[2]

1. TRUMPP. Archiv für Hygiene (Health Records), 1898.

2. A similar observation could be made regarding the findings of Mr. Roger (General Science Review, 1896) which relate to the changes undergone by by the oïdium albicans when this microorganism is subjected to the influence of an active serum.- In an article which appeared recently M.M. Kraus and Seng (Wiener Klin. Wochenschrift--Vienna Clinical Weekly--1899, No. 1) made the same criticism of the observations of Mr. Trumpp and Mr. Roger.

Red corpuscles agglomerated by a serum which comes from a different animal species appear to us to have maintained their normal aspect; they also stay normal when they are subjected to the action of an active antihematic serum, which prior exposure to a temperature of 55° had divested of alexin, dissolving without altering the agglutinin. As for the rest, the hypothesis that these different elements, the various microbes and red corpuscles, would all undergo the same change in contact with an active serum, has little likelihood to it. The existence of this viscous change is even less probable when it acts not only on organic elements, microbes, red corpuscles, but on particles of a chemical substance, such as the casein of milk. One can obtain a serum which "agglutinates milk", that is to say, which lumps the casein particles.

Would one say that the surface of these particles has become sticky and viscous under the influence of the active serum? Would one admit that it is by means of this viscosity that they can adhere to each other? One would also have to accept on this account that particles of clay in homogenous suspension in distilled water become equally covered by a special viscous and sticky coating when one adds a little sodium chloride to the liquid in which they are suspended. One knows that the presence of salt in water which contains finely ground clay in suspension is enough to provoke the formation of flakes which soon settle to the bottom of the container; it is a fact which geologists appreciate to be important and which, because it favors sedimentation, plays its role in the physics of the earth.

The existence of this adhesive material, which is the basis of the hypotheses of Mr. Gruber and Mr. Dineur, seems to the latter author to be corroborated by a significant experiment. Mr. Dineur verified that if one sets into sufficiently slow passive motion an emulsion of microbes to which the specific serum is added, the aggregation of microorganisms is greatly enhanced. The clumps are formed and increased very rapidly. Mr. Dineur supposed that rolling the microbes about on each other enhanced their joining, tangled their cilia and permitted the sticky matter with which the cilia were covered to be put into effective action, and assured definitive adhesion.

The fact observed by Mr. Dineur is accurate, but the interpretation he came up with does not seem to be. In effect, the enhancing influence of passive movement on the constitution of the flakes is also observed, with lots of evidence, in non-organic, inert precipitates. If one pours one drop of serum from about 5 to 6 cc of 0.7% sodium chloride solution, and adds nitric acid, an albuminous cloud is formed which, left to itself, only agglutinates slowly. But if, just before the formation of the precipitate, one pours a small amount of this liquid into a tube held nearly horizontally, and if one sets this tube into a slow

gentle swaying motion which is communicated to the spread-out liquid, one finds that after a few minutes the precipitate agglutinates in small white grains floating in the liquid, which has become clear. On the other hand, the liquid left motionless maintains a homogenous cloud for rather a long time. The contrast between the two tubes is striking. One can do the same experiment with other albuminous precipitates. (casein precipitate which arises in milk serum under the influence of nitric acid). The phenomenon which Mr. Dineur insists upon is also observed very vividly in the agglutination of milk by the active serum for that liquid.

Mr. Dineur's observation, far from arguing in favor of Gruber's theory, thus establishes an analogy, from the point of view of agglutination, between microbes and non-organic particles. There is another, more significant one.

It is known that the aggregation of precipitates is often governed by what appear to be minimal causes, among which has sometimes been shown to be the presence of salt in solution in the liquid. A clear example is furnished by the dispersed clay which remains in fine and homogenized emulsion in distilled water but which agglutinates and settles rapidly in water which contains sodium chloride. Therefore, if one admits that the agglutination of microbes is therefore free from the laws of molecular adhesion, one can suppose that the salts are not without influence in the phenomenon. This is what the experiment proves.

Add some cultures of cholera vibrio, aged 24 hours, to the 0.7% solution of sodium chloride (10 cc of liquid for one culture). The well-homogenized emulsion obtained is added to an energetically agglutinated dose of cholera serum. The microbes rapidly form flakes which settle at the bottom of the tube. Centrifuge them. Pour off the clear liquid which floats on the surface, so that there remains at the bottom of the tube only the compact deposit of agglomerated microbes; add the microbes to a small amount of water and a rather thick emulsion is thus obtained. Divide this emulsion into equal parts, in two tubes. Fill the first with distilled water and the second with the 0.7% solution of sodium chloride. Recentrifuge, after having shaken well and thinned. One soon verifies that, under the action of the turbine, the microbes settle more quickly in the tube with the saline solution than in that into which distilled water was poured. When the deposits are formed, retrieve the two tubes, pour off the liquids which are floating on the surface, replace them with identical liquids: one part saline solution, the other part distilled water. Shake well to loosen the microbes.

One sees that these clumps collect rapidly in the tube containing 0.7% solution of sodium chloride; on the other hand the microbes remain indefinitely scattered in that containing the distilled water. But if one withdraws a certain amount of clouded liquid from the second tube, if , for example, one transfers 10 cc to a new tube, and then adds 0.07 gr. of sodium chloride, one sees that in this distilled water to which 0.7% sodium chloride solution has been added, agglutination reappears and the decanting of microbes takes place.[1]

What is observed in the specific agglutination of the cholera vibrio is also verified by that concerning the agglomeration of that vibrio by new animal sera. We saw three years ago that new horse serum agglutinated with very real energy the cholera vibrio and other microbes (typhus, coliform and tetanus bacilli). The foregoing experiment, no longer made with specific serum but with new horse serum acting on the cholera vibrio, gives quite corresponding results.

It is not even necessary, in this case, to remove the traces of sodium chloride by repeated washing. On merely centrifuges the saline solution loaded with agglomerated microbes; one then decants the liquid, leaving only the deposit, which is then divided into two parts which are placed in two tubes; one refills one of these tubes with distilled water and the other with the sodium chloride solution. After shaking well, the agglutination is seen to reappear by itself in the presence of salt.[2]

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1. It should be noted that this "reagglutination" of microbes in distilled water to which salt has been added, does not take place quite as quickly as in the tube in which the microbes have always been in contact with the saline solution -- especially when the contact with the distilled water has been quite prolonged. It is probable that, to agglutinate well, the microbes ought to be kept in water with a certain amount of salt, which is only removed and replaced after a certain time.

2. The existence of an agglutinating power in the sera (even in new sera) has doubtless invalidated many researches carried out on the subject of the bactericide property of body fluids. Many observers have used, to evaluate the destructive power of a serum for certain microbic species, the method of successive insemination, onto gelatine, of small amounts of this serum in contact with the microbes. Now, any kind of serum inseminated with a small dose of microbes can agglutinate them in lumps which, immediately transplanted into gelatine, give only one colony each. The source of error naturally has great importance in research carried out on easily agglutinable microbes like the typhoid fever bacillus.

This experiment could be repeated, with the same result, using new horse serum and the typhoid fever bacillus.

It could also be done, this time using not only the microbes but the Kraus precipitate obtained by mixing the cholera serum and an old filtered culture of vibrios. The precipitate is subjected to the procedures described above: one verifies that it agglomerates much more strongly in liquids which contain salt than in distilled water.

It is useful to reporduce, concurrently with these experiments and following the same technique, the phenomenon historically known as the agglutination of an emulsion of fine clay, which is obtained by thinning the clay in distilled water, then filtering through paper which only lets the finest particles pass. The tubes which do not contain salt remain clouded for days. The liquids to which 0.7% sodium chloride solution has been added show a very energetic agglutination and rapid settling. Nothing is more striking than the similarity of appearance between the still-floating clumps of agglutinated microbes and the whitish flakes of clay, suspended in the liquid, which slowly settle to the bottom of the vessel.

These experiments on the absence of agglutination in distilled water thus clearly affirm the idea that agglutinin assists in provoking in elements, even though isolated at that instant, changes such that their properties of molecular adhesion become comparable to those which clay particles presented from the beginning. Consequently, in one case after another, merely adding salt suffices to effectively bring about the physical phenomenon -- thereafter possible -- of agglutination.

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One has certainly frequently accepted the existence of true bactericide properties when it has to do simply with agglutinant actions. It is even probable that one has sometimes attributed, because of the same mistake, certain properties of the agglutinins to the alexins (so-called bactericide matter). Mr. Buchner admits, for example, that alexin loses its activity, at least to a large extent, when it is mixed with distilled water. Now, the presence of distilled water in a serum should diminish in appearance the bactericide property of the latter because it weakens the agglutination and at the same time tends to increase the number of colonies which will grow on the gelatine after insemination. We have been able to verify that alexin acts very well in a medium that is poor in salt: the vibrios that have been innoculated with preventive serum but have been washed and are in suspension (without agglutinating) in 15 parts of distilled water, are transformed into granules (thus revealing the action of the alexin) when one part of new serum is added to these 15 parts of emulsion.

There, before our eyes, is a confirmation of the hypothesis we have published and brought to the attention of the reader.

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The phenomena of agglutination, thus conceived, can lead to interesting generalizations if one projects onto them the insight of ideas published by Mr. Duclaux on coagulation. What, in effect, is agglutination? It is the amassing into clumps of previously scattered organic particles which have been impressed by a special influence and whose properties of molecular adhesion have been changed. What precisely is coagulation, according to Mr. Duclaux? It is the joining into groups of particles -- which can, moreover, sometimes be extremely diffuse, presenting the appearance of a solution even in a liquid -- of particles whose relationship of molecular adhesion, on the one hand with the liquid, and on the other hand with neighboring particles, have been modified under a certain influence. With the intervention of this influence, the liquid remains homogenous. This influence is felt,"the state of equilibrium between the weight and the molecular forces is upset and either the adhesion between the liquid and the solid is diminished, or, which is more probable, the force of attraction between the particles of the solid is increased, they join into increasingly voluminous aggregates, which become visible to the naked eye, and they precipitate.[1]"

It is these changes in molecular adhesion that cause the particles (of which the chemical nature may, in the various cases, be very different), so fine that they cannot even be seen under a microscope, to join into clumps which are, at first, invisible and which cannot be revealed to the eye, growing larger then and clouding the liquid by the same phenomenon of progressive accumulation, until they constitute voluminous groupings born of increasingly abundant molecular condensation.

We do not have to follow Mr. Duclaux through the regular development of that idea, nor remark that that work of unification created, by the study of the phenomena, this concept which is capable of very closely binding facts whose close relationship has never before been suspected.

A similar bond connects the facts of agglutination to the phenomena of coagulation thus conceived. The agglutination of bacilli is due to a change in the relationships of molecular adhesion between the bodies of the bacilli and the liquid which contains them. As Mr. Duclaux put it, this phenomenon "in its entirety as in its details, reminds us of what we have seen and described in the chapter devoted to phenomena of coagulation.[2]"

1. Duclaux, Treatise on Microbiology, Vol. II, p. 263

2. Duclaux, Ibid; p. 706

Consequently, if we have the right -- as Mr. Duclaux confirms -- to consider agglutination as being a phenomenon of coagulation; if we are authorized to give henceforth to the active substance of the serum, not only the name agglutinin, which merely indicates that fact itself, without prejudice to affinities or causes, but the more suggestive appelation of "coagulin" -- we can presume that the organisms, because of their functional plasticity, the multiplicity of their resources, will be able to elaborate, if given the opportunity, active agglomerant principles not only this time, for organic elements, but with regard to characteristic chemical substances, which for some time have been recognized as having the quality of being coagulable.

This is a supposition which is verified by experience. If one gives rabbits frequent interperitoneal injections of milk (which has been heated for one hour at 65° to at least partially sterilize it), one can, within a certain amount of time, obtain a serum with special properties for milk.

Place in a tube a certain amount (3 cc for example) of this serum. Put into other tubes, for comparison, equivalent amounts of various samples of new rabbit serum. Place in the various tubes a not-too-strong dose of milk, 10 or 15 drops for example. One soon sees that the tubes containing the new serum remain opaque, white and homogenous due to the presence of the milk. In the tube containing the active serum one sees the rapid birth of first fine then rapidly growing grains and the formation of thick flakes. Soon the liquid separates into two parts, one which becomes entirely clear, the other containing the agglomerated flakes. Sometimes the flakes descend to the bottom of the vessel and it is the upper portion that becomes clear. This occurs when one uses milk which has been passed 2 or 3 times through a filter paper[1] and is thus divested of a part of its fatty globules. If one uses milk rich in fat, the flakes reach the upper part of the liquid, drawn by the lightness of the fatty globules which they have incorporated.

If one pours through paper filters such mixtures of milk with new serum, on the one hand, and "lactoserum" on the other, this second liquid passes immediately quite clear, entirely divested of the whitish cloud which the milk had passed to it; liquids based on new serum pass with an intense cloudiness.

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1. The experiments are perhaps more vivid if one uses a milk thus filtered. The milk is, in effect, cleaner and doesn't stain white the glasses it has touched, thus rendering the liquids more opaque; one can therefore more clearly judge the appearance of the agglutination.

Microscopic examination of the mixtures of milk with the sera shows that it is produced, under the influence of the lactoserum, from very abundant granular islets which are not found in the mixtures of new serum and milk. This precipitate in islets is quite similar to the casein curds resulting from the action of rennet on milk[1]. Certain aggregates are uniquely composed of a fine granular precipitate; others incorporate a large number of fatty globules which they imprison within their substance.

If to lactoserum one adds an amount of milk a bit greater than what it can agglutinate, it forms only one very abundant deposit of agglomerated matter. If one waits until all the clots are deposited, even the very smallest, when this occurs one finds that the liquid floating on the surface has become almost entirely clear, and the eye can see nothing in it. Separate by decanting, then add new serum, and there is no cloudiness; the liquid remains very transparent. If lactoserum is added a light cloudiness soon begins, which rapidly becomes accentuated; finally flakes form and make a deposit which can become abundant. This lactoserum has agglomerated extremely separated casein, scarcely clouding the liquid, and which had escaped the agglutinant action of a first (insufficient) dose of active serum.

A similar experiment can be done in the following manner: we said above that the newly-drawn serum added to milk (for example 4 cc of serum containing 10 drops of milk) passes cloudy through a filter paper. But if one repeats the filtration through the same filter paper a certain number of times the liquid becomes very clear and ends up with only a barely perceptible opalescence, which could easily pass unobserved. This liquid, examined under a microscope, is very poor in fatty globules, which one finds only rarely and in very small samples, and no other visible elements are discovered there. If one mixes a certain dose with an equal amount of new-drawn serum, no phenomenon is produced. Mix in an equal dose of lactoserum and the liquid, which was first transparent, rapidly clouds and soon white clumps of casein agglutinate in great volume which, under the microscope, reproduce the same granular islets identical with the bits of clot formed under the influence of rennet.

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1. We do not want to deduce, of course, that the agglutinin of lactoserum is identical, by its nature, to rennet. There are very great differences between these substances. The action of agglutinin depends much less on temperature than does that of rennet; it acts at low temperatures, at which rennet is almost inactive. Our serum does not have, in small doses, the property which rennet possesses of aggregating in clots truly enormous amounts of casein in suspension. On the other hand, rennet hardly ever produces a verifiable effect in a mixture of a strong dose of new-drawn serum and a small amount of milk, as long as, under these conditions, agglutination appears if lactoserum is added to the mixture.

One could also cause the formation of similar precipitates in a serum of milk obtained by the sufficiently prolonged action of rennet on milk. This serum filtered through paper is barely opalescent, it does not cloud on contact with the new-drawn serum; under the action of lactoserum, it gives an intense cloud which soon condenses into flakes. It is known that the milk serum still contains the casein which has escaped the action of the rennet, but which gives a voluminous precipitate on addition of an acid.

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Can we not now establish an analogy between the appearance, under the influence of lactoserum, of flaky precipitates in clear liquids where extremely separated casein only reveals its presence by a barely perceptible opalescence, and the production of precipitates which are born in a mixture of the two sera under the conditions indicated above? Our active serum from a rabbit that had been injected with chicken blood caused an abundant percipitate in the chicken serum: can one not admit that, in this case too, the active serum being used joins the molecular groupings, previously so sparce and dissociated that they did not cloud the clarity of the liquid? The precipitates produced in the similar mixtures of sera thus resulted in a phenomenon of agglutination[1], or, if one wishes, of coagulation, but here we no longer know which of the two terms we should use.

On the other hand, shouldn't one establish an analogy between the appearance of these precipitates, and the agglutination of microbes or of corpuscles, the condensation in voluminous clumps of previously uniformly dispersed particles?

1. The following fact corroborates that view: The property (characteristic of the active rabbit serum) of causing a precipitate in chicken serum is weakened if this active serum has previously been brought, for 1/2 hour, to a temperature of 65°; it disappears if it is heated for the same amount of time at 70°. Now, after heating to 65°, and even more so after heating to 70°, the serum has also lost to a great extent, the agglutinant property it had for the red corpuscles of chickens. As regards the action of heating, the precipitant substance thus acts as an agglutinin. On the other hand, the precipitable substance of checken serum perfectly resists heating for 1/2 hour at a temperature of 75°; a chicken serum heated in this fashion then precipitates under the action of active rabbit serum.

The one fact that distinguishes the phenomena in all these diverse cases is that the agglutinable particles are, in certain examples, so finely divided that they do not, by their condensation, change the clarity of the liquid; on the contrary, they are sometimes so voluminous (as the microbic or hematic corpuscles) that they give the liquid, before any aggregation, a cloud visible to the naked eye.

But that variation in the original size of the particles involved is only an accessory circumstance and does not enter into the concept of the very essence of the phenomenon. The distinction it creates is only secondary and in no way changes the conclusion, that nothing fundamentally separates the phenomena of agglutination and those of coagulation.

The coagulation of clay, for example, is bound to the coagulation of milk; thanks to the ideas of Mr. Duclaux, it is bound up in the agglutination of microbes by the experiments on the role of sodium chloride; besides, the agglutination of microbes is bound to the coagulation of milk because of the existence of the agglutinant lactoserum which, in liquids, can provoke coagulations comparable to the precipitations observed in the mixture of new-drawn serum with a particular specific serum, this latter itself obtained by applying to animals a system of injections entirely comparable to those which furnish the sera capable of agglomerating the microbes. Thus the links are multiplied between all these phenomena, and that is why we are forced to attribute to all, given the analogies which unite them, the same common and general explanation, attributing the appearance of agglutination to the changes in molecular adhesion.

If, to choose one example from among many different but similar examples, we return to the specific agglutination of microbes, we would have to admit that the agglutinin which affixes itself to the microbes acts to modify the bonds of molecular attraction which unite the microbic particles with their neighbors, on the one hand, and with the liquid medium on the other. The agglutinin only touches certain determined microbic species. During this first period the notion of the nature and the very organic constitution of the microbe comes to light with a great deal of evidence. It may even be, because the microbes are very sensitive to the action of the agglutinin, that they are in a sufficient state of integrity that they tend to prove certain experiments of Mr. Malvoz[1].

1. Malvoz, Research on the agglutination of the typhus bacillus. The Annales, July 1897.

But, as soon as the effect produced on molecular adhesion is obtained, the microbes aggregate as if they were inorganic particles, without it afterward being necessary to bring up again the notion of their proper organization, without it being indispensible, above all, to admit that the microbes adhere to each other, like a label stuck on a bottle, by means of a special adhesive material, with which the cilia or the swollen membranes are coated. This phenomenon of rapprochement of particles under the influence of a change in their relationships of molecular attraction should by definition be classed among the phenomena of coagulation as Mr. Duclaux characterized them.

We repeat, that view divides the total phenomenon of agglutination into two very distinct parts. In the first, the still-separate microbes are touched by the agglutinin, which fixes them; they undergo, because of this, modifications in their properties of molecular adhesion. In the second, these modifications provoke the agglutination itself.

This distinction into two periods is neither artifical nor meretricious. One can, in effect, dissociate the two phases, causing the first to appear without triggering the second. This was revealed by the experiment cited above, which places in evidence the role of sodium chloride. The washed microbes, in suspension in distilled water, have been impressed by the agglutinin; they are immobilized (we should add here that they have, moreover, become very sensitive to the action of alexin) and they are prepared to be agglutinated with energy. But it is necessary, in order for the second phase of the phenomenon to develop, to introduce a bit of sea salt into the emulsion, necessary for agglutination itself.

As for the Kraus phenomenon, his interpretation is immature. It has never been demonstrated that the Kraus precipitate has anything to do with the true agglutination of microbes; it may be, in effect, that this precipitate is comparable to that which one obtains by mixing defibrinated chicken blood with active rabbit serum (previously injected with chicken blood), and which does not appear to have any relationship to the agglutination of the corpuscles themselves. But if the precipitate identified by Kraus were formed of the true agglutinable matter of microbes, it would, it seems to us, approach, from the point of view of its method of production, the casein precipitates that lactoserum produces in media (filtered milk serum, for example) where that substance is so divided that it doesn't cloud the clarity of the liquid at all.

This precipitation thus would represent, in this hypothesis, an agglutination of very dispersed microbic material.

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The idea that the agglutination of the indicated elements, corpuscles, microbes or inorganic particles, casein, has the characteristics of the phenomenon of coagulation should suggest some thought as to the significance of the active properties of the serum.

To begin with we will list briefly, without repeating in detail the text we submitted above, the essential properties, verifiable in vitro, that we have recognized of the specific sera or, to be precise, the two sera we have taken as typical, the cholera serum and the active serum for rabbit corpuscles (serum coming from guinea pigs injected with rabbit blood). These sera have rather similar properties and we have recently stressed their analogies. These properties are the following:

1. These sera agglutinate the indicated elements, suppressing the mobility with which they may be endowed.

2. When the vibrios or the corpuscles have been in contact with the sera, they become more likely to experience the altering and destructive influence of the alexin (alexin or lysin being, as is known, the bacteria- and corpuscle-destroying material which itself is destroyed at a temperature of 55°, and which can act as a sort of dissolvant enzyme on certain delicate elements, vibrios, corpuscles). These two properties are also found in sera which have been heated to 55° or 60°.

3. These sera (in a cool state) possess alexin, and that is why, when added to elements to which they have been sensitized, they profoundly alter them (transformation of the vibrios into granules, destruction of corpuscles) and even cause them to be subjected to the phenomenon of partial dissolution.

If we were to list only those properties that are really characteristic of these sera, we could eliminate the third, that of possessing alexin. In effect, that property also appears in new animal sera. After causing the alexin in the specific serum in question to disappear by means of heating to 55°, after having thus destroyed its bacteria- or corpuscle-destroying power, the alexin can be restored and this power can be re-established by adding a bit of new serum to the liquids[1].

Thus there remain two properties which, to tell the truth, are still found to a certain degree in the new sera but only weakly represented. Thus because they are very intense they can be considered as characteristic of the sera from vaccinated animals.

We have not taken up here the question of knowing if these two properties are due to the presence of two different substances or if they can be attributed to the activity of the same material; we will wait and return to that question in a subsequent report. In spite of this, the most remarkable of these two properties is that of sensitizing the elements to the action of the alexin.

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1. For the details of these experiments on the cholera vibrio, see our article which appeared in the Annales, June 1895; for that concerning the corpuscles, see the issue of October 1898.

When we say that there is a sensitizing substance in the specific sera, that merely implies that the sera act directly on these elements. This sensitizing substance has, in effect, a very special predeliction to attach itself to the elements which it can impress.

Cholera vibrios transported in sufficient quantity in a liquid containing cholera serum absorb their active principles. If they are centrifuged and the clear surface liquid is decanted, one finds that this liquid has lost, along with its agglutinizing power, the ability to sensitize new microbes to the action of the alexin. In other words, the new vibrios, placed in contact with the liquid, are neither immobilized nor agglutinated and can be injected into the peritoneum of a guinea pig or mixed in vitro with the new serum without being transformed into granules. The same phenomenon of absorption, or fixation, is produced if the vibrios are grown in a bouillon to which a not-very-strong amount of cholera serum has been added. The microbes which develop divest the liquid of all its special properties at the same time[1]. The same facts are established with regard to active sera for red corpuscles. These, placed in contact with the serum, absorb the agglutinant substance as well as the sensitizing substance[2]. The liquid which one separates by centrifugation of such a mixture is inactive on new corpuscles. We add that the agglutinins of new sera are equally absorbed

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1. The result of our experiments is in complete contradiction with the facts observed by Mr. Pfeiffer (Zentralblatt für Bakteriologie--Central Newspaper for Bacteriology--1896), who did this last experiment. Mr. Pfeiffer established that there is absorption of the agglutinin, but he declared that the liquid separated from the microbes which were proliferated keeps the property of provoking the phenomenon of granular transformation if it is injected, mixed with new vibrios, into the peritoneum of a new guinea pig. Moreover, Mr. Pfeiffer's conclusion, according to which the active sera do not act (on the microbes) in the same manner in vitro as in the peritoneum, is erroneous. The action in vivo and in vitro is the same. Experience shows that the minimum dose of cholera serum that must be introduced in an emulsion of vibrios to transform them into granules when they are placed in contact (by injection in the peritoneum) with the alexin which is contained in the peritoneal exudate, is identical with that which should be made to act on the vibrios to transform them into granules, in vitro, when they are placed in contact with the alexin of new serum. The direct action which the serum exercises on the microbe in the peritoneum and in vitro is the same. This minimum dose is also, for the vibrios, very close to the minimum agglutinating dose. We will return to these questions later.

2. We should note here that Mr. Ehrlich (Berliner Klin. Wochenschr.--Berlin Clinical Weekly--1899, No. 1) has recently seen that an antihematic serum worn out by contact with the corpuscles becomes incapable of forming, with new serum, a mixture which dissolves new corpuscles.

by the corpuscles or the microbes[1]. All this shows us that the organisms, during the course of vaccination, differ in that they produce not very strong quantities of this "dissolving enzyme", alexin, but substances which enhance the action of this enzyme, principles capable of becoming fixed on the elements and sensitizing them to the influence of the alexin.

Thus it is above all because of these enhancing substances that the sera of vaccinated animals are capable of producing well-defined phenomena of digestion if the elements affected do not present too great a resistance, which unfortunately is the case for a large number of microbes[2]. Thus we could consider such sera as analogous to digestive juices.

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1. Apropos of this, let us mention a few words about a very curious experiment which, to tell the truth, is a bit off the subject of this report. If one places a certain amount of new serum which possesses very strong agglutinating properties (new horse serum) in an emulsion of cholera vibrios, one finds a certain agglutination. If it is centrifuged and the clear liquid separated off, one finds that this liquid no longer agglutinates the cholera vibrio but it still strongly agglutinates the typhus bacillus. Inversely, if a new amount of this same new horse serum is placed in contact with an emulsion of typhus bacilli, one finds that the liquid decanted after centrifugation has become incapable of agglutinating the typhus bacillus, but it still strongly agglutinates the cholera vibrio. Thus it seems rather certain that these two different microbes take two different agglutinins from the same serum. It seems that the specificity of the agglutinins which clearly characterize the sera of vaccinated animals, already exists in a germinal state in the new animal. From such experiments we can without doubt clarify a bit the obscured question of the origin of the specificity of the active properties of sera. One might imagine that vaccination against a given microbe is accompanied by the production of a large amount of that special agglutinin, which pre-exists in a weak dose.

2. It is known that the typhus bacillus, the coliform bacilli, for example, show granular transformation only with difficulty, even in the presence of an active serum. Many microbes are even less sensitive to the bactericide properties.

This analogy which is sketched between the properties of active sera and of digestive juices is verified if one considers that the active substances of the serum probably have as their sources the digestive cells which Mr. Metchnikoff discovered which play a very important role in immunity, and which, in their evolution, attach themselves to those amoeba-like cells which are capable, because of the simplicity of their organisms, of assuring the nutrition of the individual, thanks to their functions of intracellular digestion. There are similar cells, found by Mr. Metchnikoff in the hierarchy of nature, which assume the entire digestive function in species which are not highly differentiated. Even more, these amoeba-like cells represent the origin of our digestive apparatus, because Mr. Metchnikoff has established that digestion, at first uniquely intracellular, with evolution becomes extracellular, inasmuch as these cells acquire the property of outwardly secreting their dissolvant juices.

The source of the agglutinins, of the sensitizing substances, is not defined, it is true; but as for alexin, the so-called dissolvant principle, numerous observations indicate that it comes from a leucocyte origin.

We do not want to speak in absolute terms about a subject which, though still clouded in such obscurity, is nevertheless being considered at the present time. Nevertheless, important characteristics join the active sera and the digestive juices, and the analogy could be explained if, as various facts and serious presumptions make it likely, the production of the active substances were to be attributed to the phagocytic system, to that group of cells which possess a high degree of digestive properties which they have preserved intact throughout their evolution.

If the facts presented here are at all persuasive, one will come to the conclusion that immunity appears more and more, not only from a biological point of view (which is solidly established at the present time) but also from a chemical point of view, to be a particular case of the general physiology of digestion.

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## CONCLUSIONS

I. The theories which explain microbic agglutination by swelling and viscosity, whether of membranes or of cilia, run into numerous objections and do not explain all the phenomena of agglutination. It is just like the theory which recognizes as the cause of agglutination the formation of a precipitate in the liquid.

II. Agglutination can take place in very different elements (corpuscles, microbes, casein). There must be a single explanation for the various cases of agglutination by the sera.

III. It may be admitted that the agglutinins, in affixing themselves on the agglutinizable elements, bring about modifications in the molecular attractions which unite these elements either with each other or with the liquid medium. The total phenomenon of agglutination should be divided into two phases, the first of which can be produced experimentally (period of impression for the agglutination of still-isolated elements) without provoking

the second (period of agglutination proper). In the first phase alone, the very nature of the elements intervenes. In the second phase the particles, obeying the molecular attraction, may by their agglutination present peculiarities that are found in the aggregation of mineral particles.

IV. The phenomena of agglutination closely resemble the phenomena of coagulation.

V. The phenomena of true agglutination can be provoked in clear liquids in which the particles are extremely dispersed.

VI. Active sera and digestive juices can be compared to a certain extent, from the point of view of their coagulant and dissolvant properties.

VII. Following a conclusion already formulated in a preceeding article about antihematic sera, the production, by organisms undergoing vaccination, of substances which are noxious for microbes, should not be interpreted in a teleological sense. It is not with the intention of defending itself that the organism makes these noxious substances. It simply uses its pre-existant functional capacities against the microbes, also capable of being exercised, should the need arise, against non-dangerous elements such as red corpuscles or the casein of milk. The special properties of the sera of vaccinated animals exist in a germinal state in the new sera. It seems that this also ought to concern the specific character presented by the sera of vaccinated animals.